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Award Number: DAMD17-98-1-8317

TITLE: Secretory Heat Shock Protein - gp96-Ig Chaperoned her-
2/new Vaccines

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REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 99 - 30 Jun 00)		
4. TITLE AND SUBTITLE Secretary Heat Shock Protein - gp96-Ig Chaperoned her-2/new Vaccines		5. FUNDING NUMBERS DAMD17-98-1-8317		
6. AUTHOR(S) Eckhard Podack, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Miami School of Medicine Miami, Florida 33101 E-MAIL: epodack@miami.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The hypothesis that secreted heat shock protein gp96 will trigger an immune response was tested in a tumor system in experimental animals. It was found that gp96 is able to induce tumor specific CD8 CTL without the help of CD4 cells. The postulated molecular and cellular mechanism involves the following steps: 1) gp96-Ig peptide secretion; 2) Binding to endocytosis receptor on dendritic cells; 3) Dendritic cell activation/maturation, including up-regulated expression of CD80/86; 4) Uptake of gp96 and routing of peptides to endoplasmic reticulum; 5) loading of trimmed peptides on class I MHC molecules and transport to the cell surface; 6) CD8-pre-CTL activation (independent of CD4 T cell help).				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 23	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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Introduction:

The heat shock protein gp96 is normally resident in the endoplasmic reticulum and serves as a chaperone for peptides being sorted for MHC class I loading and transport to the cell surface. The strategic location and function of gp96 in the class I pathway makes it an ideal target for genetic engineering for the purpose of generating tumor specific CTL responses. The current work is concerned with studies converting gp96 with its associated peptides from an endoplasmic reticulum resident protein into a secretory protein. Insertion of secretory gp96 (gp96-Ig) into tumor cells is postulated to increase the immunogenicity of the tumor, since it has been shown previously that heat shock proteins are highly immunogenic. In addition, insertion of secretory gp96 along with known tumor antigens such as her-2-neu into tumor cells, should result in the secretion of gp96-Ig with associated her-2neu peptides. Uptake of secreted gp96-Ig by APC (e.g. dendritic cells) is expected to render them sensitive to lysis by specific CTL.

Body:

Secreted heat shock protein gp96-Ig elicits CD8 cells for tumor rejection. Purified heat shock protein gp96 isolated from tumor cells is highly immunogenic and induces tumor specific immune responses. The endoplasmic reticulum (ER)-resident gp96 appears to chaperone peptides, including those derived from tumor antigens, on their way to presentation by MHC class I. Replacement of the ER-retention signal of gp96 with the Fc portion of murine IgG1, in this study, generated a secretory form of gp96, gp96-Ig. Tumor cells transfected with gp96-Ig exhibited decreased tumorigenicity and increased immunogenicity in vivo and were rejected after initial growth. Rejection of tumors secreting gp96-Ig required CD8 T cells during the priming and during the effector phase while CD4 T cells were not required for rejection in either phase. Carrageenan, a compound known to inactivate macrophages in vivo, also did not diminish tumor rejection. Thus, immunization with tumors secreting gp96-Ig, unlike immunization with purified, tumor derived gp96, or with irradiated tumor cells, generates efficient tumor rejecting CD8 CTL without requirement for CD4 or macrophage help.

Characterization of Shared and Private Tumor Antigens Presented by HLA A1 or HLA A2 Rejection of tumors by the immune system is believed to require the participation of CD4 and/or CD8 CTL. Tumor derived peptides presented by MHC class I molecules are targets for CD8⁺ CTL. MHC restricted CD8⁺ CTL are also required for the identification and characterization of tumor antigens, including breast tumors antigens, useful for immune therapy. For many human solid tumors, however, tumor antigens remain undefined due to the difficulty of generating MHC restricted tumor specific CTL required for their analysis. Discovery of novel tumor antigens to breast tumors will greatly expand the opportunities for immune intervention in breast cancer.

CD8⁺ CTL responses are modulated by CD4⁺ helper T cells and by antigen presenting cells. In this study highly purified CD8⁺ T cells were mixed with tumor cells in primary cultures in the absence of any other cells to reduce the complexity of CTL generation and to avoid potential immunosuppressive or tolerizing effects during CTL stimulation.. Tumor cells were transfected with HLA A1 or A2 and used to stimulate HLA A1 or A2 matched CD8⁺ T cells. MHC class I

matching and omission of other cells in primary culture was highly effective in generating MHC class I-restricted CTL to poorly immunogenic small cell lung carcinomas (SCLC), used as model system in this study. Cytotoxicity was further enhanced by cotransfection of tumor cells with B7.1 (CD80). ICAM-1 (CD54) was not as effective. SCLC tumor cells were able to present tumor specific peptides with HLA A1 and A2 and were lysed by A1 or A2 restricted CD8⁺ CTL. A1 and A2 restricted CD8⁺ CTL detected shared tumor antigens on unrelated SCLC tumor lines in addition to private antigens. The use of direct antigen presentation by MHC class I transfected tumors to MHC class I matched CD8⁺ T cells is an effective way to generate MHC class I-restricted CTL towards poorly immunogenic tumors in vitro permitting the molecular identification of their tumor antigens. Characterization and cloning of tumor antigens using this approach for CTL generation is in progress.

Key research accomplishments:

- Demonstrate utility of tumor secreted gp96
- Demonstrate antigen specificity of secreted gp96
- Describe new pathway of CD4 T cell independent activation of CD8 CTL
- Postulate direct dendritic cell activation by gp96 independent of CD40 signals

Reportable outcomes:

Publications 99-00

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Publications (in press or submitted):

- Jiang, Z., Podack, E., Levy, R. Allogeneic BMT Using Perforin and FasL Double-Defective CD4⁺ Donor T Cells: A Crucial Role for Cytotoxic Function by Donor Lymphocytes Prior to GVHD Pathogenesis. 2000. Blood.
- Muta, H., Boise, L., Fang, L., Podack, E. CD30 signals integrate expression of cytotoxic effector molecules, lymphocyte trafficking signals, and signals for proliferation and apoptosis. 2000. J. Immunol.

Abstract:

- Yamazaki, K., Podack, E. Characterization of MHC Restricted CTL for the Isolation of Tumor Antigens. 2000. Era of Hope-Department of Defense Breast Cancer Research Program Meeting, Atlanta, GA.

Conclusions:

The work completed up to this point allows the formulation of the hypothesis that secreted heat shock protein gp96 is highly immunogenic by mediating direct activation of dendritic cells without CD4 help. We postulate that dendritic cells use receptors to endocytose gp96 along with its bound peptides and that receptor binding of gp96 results in the activation/maturation of dendritic cells and routing of the endocytosed peptides to the endoplasmic reticulum for loading onto class I MHC complexes. Using human dendritic cells, this hypothesis will be tested. In addition tumor vaccines and CTL assays are developed for clinical testing.

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Not applicable

Appendix:

- Yamazaki, K., Podack, E. Characterization of MHC Restricted CTL for the Isolation of Tumor Antigens. 2000. Era of Hope-Department of Defense Breast Cancer Research Program Meeting, Atlanta, GA.
- Yamazaki, K., Nguyen, T., Podack, E. Cutting Edge: Tumor Secreted Heat Shock-Fusion Protein Elicits CD8 Cells for Rejection. 1999. Cutting Edge.
- Yamazaki, K., Spruill, G., Rhoderick, J., Spielman, J., Savaraj, N., Podack, E. Small Cell Lung Carcinomas Express Shared and Private Tumor Antigens Presented by HLA-A1 or HLA-A2. 1999 Cancer Research September 1999.

CHARACTERIZATION OF MHC RESTRICTED CTL FOR THE ISOLATION OF TUMOR ANTIGENS

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Rejection of tumors by the immune system is believed to require the participation of CD4 and/or CD8 CTL. Tumor derived peptides presented by MHC class I molecules are targets for CD8⁺ CTL. MHC restricted CD8⁺ CTL are also required for the identification and characterization of tumor antigens, including breast tumors antigens, useful for immune therapy. For many human solid tumors, however, tumor antigens remain undefined due to the difficulty of generating MHC restricted tumor specific CTL required for their analysis. Discovery of novel tumor antigens to breast tumors will greatly expand the opportunities for immune intervention in breast cancer.

CD8⁺ CTL responses are modulated by CD4⁺ helper T cells and by antigen presenting cells. In this study highly purified CD8⁺ T cells were mixed with tumor cells in primary cultures in the absence of any other cells to reduce the complexity of CTL generation and to avoid potential immuno suppressive or tolerizing effects during CTL stimulation.. Tumor cells were transfected with HLA A1 or A2 and used to stimulate HLA A1 or A2 matched CD8⁺ T cells. MHC class I matching and omission of other cells in primary culture was highly effective in generating MHC class I-restricted CTL to poorly immunogenic small cell lung carcinomas (SCLC), used as model system in this study. Cytotoxicity was further enhanced by cotransfection of tumor cells with B7.1 (CD80). ICAM-1 (CD54) was not as effective. SCLC tumor cells were able present tumor specific peptides with HLA A1 and A2 and were lysed by A1 or A2 restricted CD8⁺ CTL. A1 and A2 restricted CD8⁺ CTL detected shared tumor antigens on unrelated SCLC tumor lines in addition to private antigens. The use of direct antigen presentation by MHC class I transfected tumors to MHC class I matched CD8⁺ T cells is an effective way to generate MHC class I-restricted CTL towards poorly immunogenic tumors in vitro permitting the molecular identification of their tumor antigens. Characterization and cloning of tumor antigens using this approach for CTL generation is in progress.

Cutting Edge: Tumor Secreted Heat Shock-Fusion Protein Elicits CD8 Cells for Rejection¹

Koichi Yamazaki, Timmy Nguyen, and
Eckhard R. Podack²

The endoplasmic reticulum resident heat shock protein gp96 chaperons peptides, including those derived from tumor Ags, on their way to presentation by MHC class I. Replacement of the endoplasmic reticulum retention signal of gp96 with the Fc portion of murine IgG1 generated a secretory form of gp96, gp96-Ig. Tumor cells secreting gp96-Ig exhibited decreased tumorigenicity and increased immunogenicity in vivo and were rejected after initial growth. Rejection required CD8 T cells during the priming and effector phase. CD4 T cells were not required for rejection in either phase. Carrageenan, a compound known to inactivate macrophages in vivo, did not diminish CD8-mediated tumor rejection. Therefore, immunization with tumors secreting gp96-Ig generates efficient tumor-rejecting CD8 CTL without requirement for CD4 or macrophage help. In contrast, immunization with purified, tumor-derived gp96 or with irradiated tumor cells requires both. *The Journal of Immunology*, 1999, 163: 5178-5182.

The heat shock protein (hsp)³ gp96, localized in the endoplasmic reticulum (ER), is thought to serve as a chaperon for peptides on their way to MHC class I and II molecules (1-4). Gp96-chaperoned peptides comprise the entire spectrum of peptides and larger protein fragments generated in cells and transported into the ER (5-9). Gp96 obtained from tumor cells and used as a vaccine induces specific tumor immunity (3, 10-13), presumably through the transport of tumor-specific peptides to APCs.

We developed a secretory form of gp96, gp96-Ig, and tested it in tumor models. Transfection of tumor cells with the cDNA for gp96-Ig resulted in gp96-Ig secretion. As shown in this publica-

tion, gp96-Ig-secreting tumor cells caused powerful immunization and tumor rejection in vivo dependent exclusively on CD8 cells.

Materials and Methods

Cell lines

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in medium with 10% FCS. Human small cell lung carcinoma (SCLC) cell lines (SCLC-2 and SCLC-7) were established as described (14). Chicken OVA cloned into the expression vector, apc-NEO-OVA, was kindly provided by Dr. M. Bevan (Seattle, WA) (15) and used to transfect Lewis lung carcinoma (LLC).

Construction of gp96-Ig

To generate the gp96-Ig fusion protein, the KDEL sequence was deleted and replaced with the hinge, CH2 and CH3 domains of murine IgG1 (16-23); double-stranded cDNA was prepared from Jurkat DNA (24) with the GeneAmp RNA PCR Kit (Perkin-Elmer Cetus, Norwalk, CT) and amplified by PCR. The PCR primers were 5'-ATTACTCGAGGGCCGACGC CATGAGGG-3' and 5'-GCCCGGATCCTTCAGCTGTAGATTCCTT TGC-3' (18, 19). The PCR primers included an *Xho*I site (forward primer) and a *Bam*HI site (reverse primer). The hinge, CH2 and CH3 domains of murine IgG1, was amplified by using murine IgG1 cDNA as a template and mutating the three cysteines of the hinge portion to serines (21, 25). The PCR primers were 5'-GCGAGGATCCGTGCCAGGGATTCTGGTT CTAAG-3' and 5'-CTAAGCGGCCGCAAGGACACTGGGATCATTT ACCAGG-3'. The PCR primers included a *Bam*HI site (forward primer) and *Nor*I site (reverse primer). Gp96 was inserted into *Xho*I and *Bam*HI sites of the eukaryotic expression vector, pBCMGsNeo and pBCMGHis (26-29), and transfected into SCLC-2, SCLC-7, B16F10, MC57, LLC NIH3T3, EL4, E.G7, and P815. Transfected cells were selected with 1 mg/ml of G418 or 2.5-10 mM of L-Histidinol (Sigma, St. Louis, MO).

ELISA

This was conducted using Abs to the Ig tag. Gp96-Ig-producing cells were plated at 10⁶/ml in AIMV or IMDM with 10% FCS, and culture supernatants were harvested at different time points. For analysis of intracellular expression of gp96-Ig, cells were lysed by three freeze-thaw cycles and centrifuged 60 min at 13,000 × g (30).

Purification of gp96-Ig fusion protein

Gp96-Ig was purified by affinity chromatography on a protein A column using standard procedures (Bio-Rad, Hercules, CA) (31). The concentration of gp96-Ig was determined by the Micro BCA protein assay reagent kit (Pierce, Rockford, IL). SDS-PAGE and Western blotting were done using a standard procedure.

FACS analysis

For membrane staining of gp96-Ig-transfected SCLC, cells were stained with goat anti-mouse IgG-FITC or goat anti-rabbit IgG-FITC as a control for 15 min at 4°C and analyzed by a Becton Dickinson FACSscan flow cytometer (San Diego, CA). For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with 1% saponin followed by staining with goat anti-mouse IgG-FITC, goat anti-mouse IgG-PE, goat

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Received for publication August 12, 1999. Accepted for publication September 15, 1999.

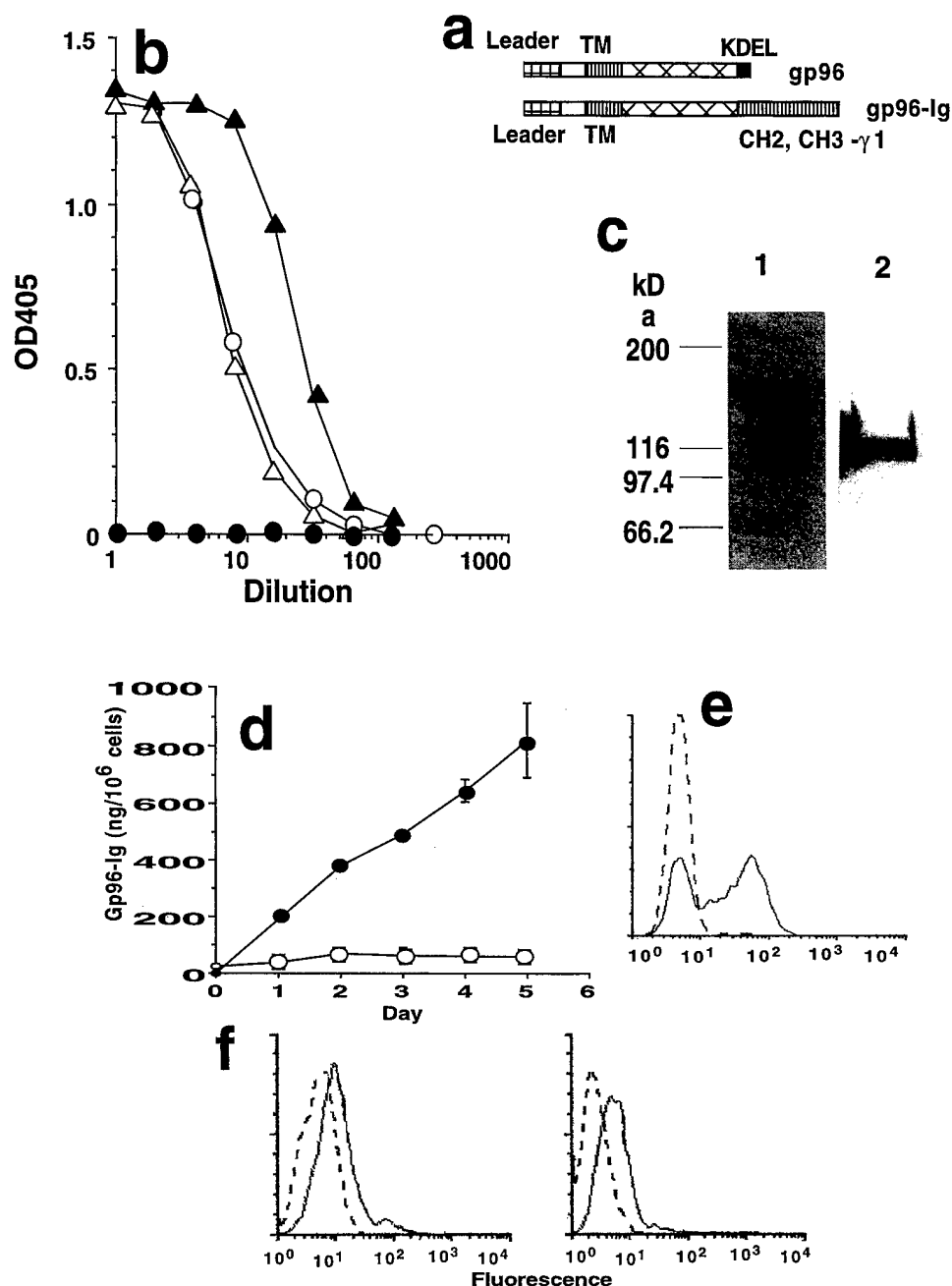
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¹ This work was supported by National Institutes of Health Grants CA590351, CA39201, and CA80228 and Department of Defense Grant DAMDS17-98-1-8317.

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³ Abbreviations used in this paper: hsp, heat shock protein; ER, endoplasmic reticulum; gp96-Ig, secretory form of gp96; SCLC, small cell lung carcinoma; LLC, Lewis lung carcinoma.

FIGURE 1. Characterization of gp96-Ig. *a*, Schematic representation of the gp96-Ig construct. *b*, ELISA for gp96-Ig of supernatants from gp96-Ig cDNA-transfected and untransfected SCLC-7; cells were plated at 10^6 /ml, and supernatants were harvested on day 3 and day 6; purified mouse IgG (500 ng/ml) served as standard. \circ , Murine IgG standard (500 ng/ml); \triangle , SCLC-7 gp96-Ig on day 3; \blacktriangle , SCLC-7 gp96-Ig on day 6. *c*, SDS-PAGE of protein A-purified gp96-Ig. Lane 1, Coomassie blue stain (1μ g protein); lane 2, Western blot with monoclonal anti-gp96-Ig (anto-Grp94, 9G10) (100 ng protein). *d*, Accumulation of gp96-Ig. \bullet , Gp96-Ig in the culture supernatant; \circ , Gp96-Ig in cell lysates. Gp96-Ig was quantitated by ELISA; SCLC-gp96-Ig was plated at 10^6 /ml. *e*, Intracellular staining of gp96-Ig by FACS analysis of permeabilized SCLC-gp96-Ig; dashed line, goat anti-rabbit IgG-FITC (negative control); solid line, goat anti-mouse IgG-PE. *f*, SCLC surface stain and SCLC-gp96-Ig surface stain. Absence of surface stain for gp96-Ig on unpermeabilized SCLC; *left*, untransfected; *right*, gp96-Ig-transfected SCLC. Dashed line in both panels is goat anti-rabbit IgG-FITC; solid line, goat anti-mouse IgG-FITC.



anti-rabbit IgG-FITC, or goat anti-syrian hamster IgG-FITC for 15 min at 4°C and analyzed by a flow cytometer.

Tumor inoculation and vaccination

Tumorigenicity *in vivo* was determined by s.c. injection of live tumor cells in 200 μ l PBS into the flanks of mice. The size of tumors was measured in two dimensions twice weekly for at least 2 mo. When mean tumor growth exceeded 10 mm diameter, the mice were sacrificed.

Mice were immunized by s.c. injection of 10^6 live E.G7-gp96-Ig or irradiated E.G7 as a control (in 200 μ l PBS), given in the right flank. Two immunizations at 2-wk intervals were given. Two weeks later, mice were challenged by s.c. injections of the indicated number of live tumor cells (ELA, E.G7, LLC, or LLC-OVA in 200 μ l PBS) into the left flank.

Depletion of T cells or macrophages *in vivo*

A total of 100 μ g of GK1.5 (anti CD4) or 2.43 (anti CD8) in 200 μ l PBS was administered by i.p. injection (32, 33). Depletion of CD4 and CD8 cells was verified by FACS analysis. CD4 or CD8 levels remained low (>95% depletion) for >2 wk following Ab injection (data not shown). For

functional inhibition of macrophages, 1 mg of Carrageenan (type II; Sigma) in 200 μ l PBS was administered by i.p. injection (32, 34).

Results

The ER-resident hsp gp96 purified from tumor cells can provide tumor-specific immunity (35). The C-terminal sequence KDEL of gp96 serves as ER retention signal. Deletion of this sequence resulted in the secretion of gp96 together with bound peptides from transfected tumor cells and may render tumors more immunogenic to allow tumor rejection by the immune system.

Characterization of secreted hsp gp96-Ig

Replacing the KDEL sequence of gp96 with the hinge, CH2 and CH3 domain of murine IgG1 (Fig. 1a), an Ig isotype inefficient in Fc receptor binding, and transfection of the cDNA into tumor cells resulted in the secretion of gp96-Ig into the culture supernatant,

Table I. Secretion of gp96-Ig into culture supernatants^a

Cell Lines	Gp96-Ig/10 ⁶ Cells × 24 h (ng)
SCLC-2	140
SCLC-7	500
NIH3T3	500
EL4	160
E.G7	60
P815	<5
LLC	70
B16F10	312.5 ^b
MC57	3,300

^a Gp96-Ig cDNA was expressed in a bovine papilloma virus-derived episomal vector under the CMV or metallothioneine promoter.

^b Metallothioneine promoter.

where it was quantitated by ELISA (Fig. 1*b*). Protein A purified gp96-Ig upon SDS-PAGE migrated with a major band of the predicted molecular mass of 120 kDa for the fusion protein and two minor, higher molecular bands previously reported also for unmodified gp96 (Fig. 1*c*) (10). Western blotting with a mAb specific for gp96 confirmed the identity of the fusion protein. Only the major band is stained, suggesting that the minor bands are glycosylation variants of gp96 not recognized by the Ab.

Secretion of gp96-Ig resulted in its time-dependent, linear accumulation in the supernatant (Fig. 1*d*). Intracellular gp96-Ig was detected at a low and constant steady-state level in lysates of transfected cells, indicating that it does not accumulate in the cell. FACS analysis of membrane-intact, transfected tumor cells revealed no staining with anti-mouse IgG above background, indicating that the Ig moiety of the fusion protein is not displayed on the outer leaflet of the plasma membrane (Fig. 1*f*). In contrast, upon permeabilization of the membrane, gp96-Ig is detected intracellularly with a goat anti-mouse IgG Ab, but not by control goat anti-rabbit IgG Abs (Fig. 1*e*). The transmembrane domain of gp96 does not interfere with the secretion of gp96-Ig and does not lead to intracellular accumulation. These data are consistent with previous reports suggesting that the transmembrane domain is not used for anchoring of gp96 in the membrane and that gp96 is not an integral membrane protein (36).

All murine and human cell lines transfected with gp96-Ig secreted the fusion protein (Table I). Mock-transfected cells did not secrete gp96-Ig. E.G7 is an OVA transfectant of the EL4 lymphoma forming lethal tumors in syngeneic C57BL/6 mice. Gp96-Ig transfection of E.G7 allows the determination whether E.G7-gp96-Ig immunizes against the EL4 parent tumor in addition to E.G7, the OVA surrogate Ag-transfected tumor. As second tumor, LLC transfected with gp96-Ig or with OVA was used because, in contrast to E.G7, it is a nonhemopoietic, low-immunogenic tumor. Both cell lines secrete comparable amounts of gp96-Ig (Table I).

Secreted gp96-Ig is responsible for decreased tumorigenicity

Secretion of gp96-Ig decreases the tumorigenicity of E.G7 in C57BL/6 mice by >100-fold when compared with mock-transfected or untransfected E.G7. Subcutaneous inoculation of 10 million hsp-secreting tumor cells caused tumors in only 10% of the inoculated mice (Fig. 2*A*). A similar reduction of tumorigenicity by gp96-Ig secretion was observed with transfected EL4 (data not shown). Gp96-Ig secretion by LLC resulted in a more moderate, ~5-fold, decrease of tumorigenicity (Fig. 2*B*).

To determine immunogenicity and immune memory responses, C57BL/6 mice were immunized twice at 2-wk intervals with a

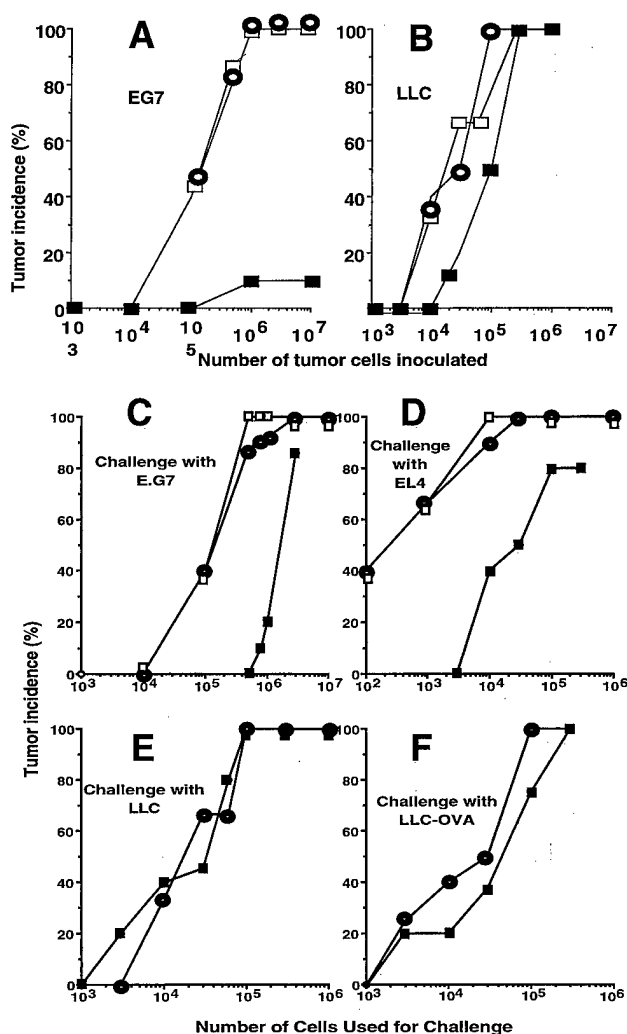


FIGURE 2. Decreased tumorigenicity of gp96-Ig-transfected E.G7 (A) and LLC (B). ■, Gp96-Ig transfected; ○, mock transfected; and □, untransfected cells. Groups of six mice were used per dose of inoculated cells. C–F, Secretory gp96-Ig vaccination generates tumor-specific memory. C57BL/6 mice were immunized twice in biweekly intervals with 10⁶ gp96-Ig-transfected E.G7 (■ in all panels), with 10⁶ irradiated E.G7 (□), or not immunized (●). Two weeks later, mice were challenged (six mice per group) with the number of tumor cells indicated in the panels. Mice not developing tumors were observed for 3 mo and then judged tumor free.

dose of nonirradiated E.G7-gp96-Ig (10⁶) that was rejected. Subsequently, they were challenged with untransfected or mock-transfected E.G7, parental EL4, untransfected LLC, and OVA-transfected LLC (Fig. 2, C–F). Mice immunized with irradiated E.G7 or unvaccinated mice served as controls. E.G7-gp96-Ig-immunized mice resisted a 10-fold higher tumor challenge by E.G7 than mice vaccinated with irradiated cells or unimmunized mice (Fig. 2*C*). Tumor growth in vaccinated mice was frequently delayed. The effect of immunization was even more pronounced when challenged with EL4, allowing a fifty-fold dose increase of EL4 challenge compared with the controls (Fig. 2*D*). As expected, E.G7-gp96-Ig immunization offered no protection against challenge with untransfected or vector-transfected LLC (Fig. 2*E*), while a moderate, ~3-fold, increase in protection was observed when OVA-transfected LLC were used as challenge (Fig. 2*F*). The strong protection of mice immunized with E.G7-gp96-Ig against EL4 challenge may be due to multiple tumor Ags shared by E.G7 and

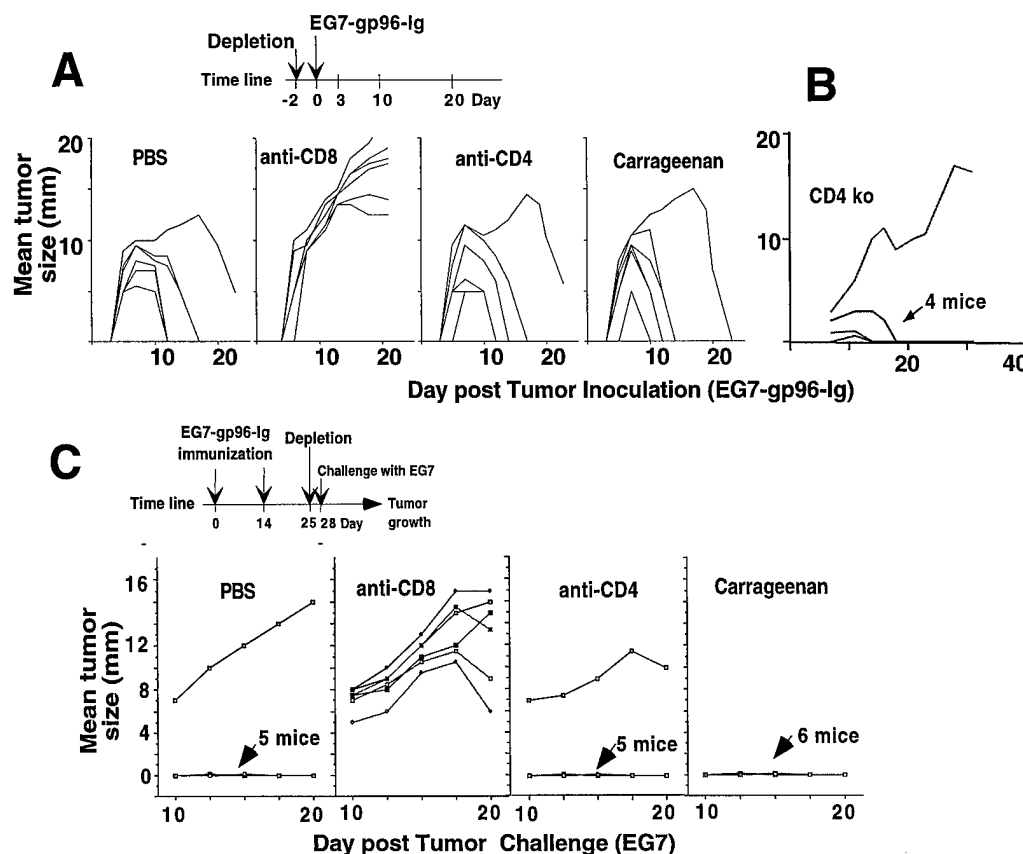


FIGURE 3. A, Effect of depletion of immunocompetent cells on the rejection of 10^6 E.G7-gp96-Ig during the priming phase; controls received PBS. Tumor growth curves of individual mice are shown. The depletion schedule is shown schematically on top. Depletion of immunocompetent cells was done with anti-CD8, anti-CD4, or Carrageenan 2 days prior to inoculation of 10^6 E.G7-gp96-Ig. B, CD4-deficient mice can reject E.G7-gp-Ig. Five CD4-deficient mice were challenged with unirradiated 10^6 E.G7-gp96-Ig s.c. Tumor growth was recorded, and the mean tumor diameter is reported. C, Effect of depletion of immunocompetent cells on the effector phase of E.G7 rejection. The schedule of immunization and immunodepletion is shown schematically on the top. For immunization, 10^6 unirradiated E.G7-gp96-Ig were inoculated twice s.c. into groups of six mice. Three days prior to challenge with 10^6 E.G7, immune cells were depleted as above; controls received PBS. Tumor growth was recorded and is reported as mean tumor diameter.

EL4. The weak protection against challenge with LLC-OVA depends on T cells recognizing a single or limited number of epitopes derived from the OVA surrogate Ag for T cell recognition.

CD8 cells are required in the priming and effector phase

The involvement of immune mechanisms in the rejection of E.G7-gp96-Ig was further examined by *in vivo* depletion/inactivation of immunocompetent cells. It has been reported that Meth A tumor-derived gp96 requires CD4 cells, CD8 cells, and macrophages for effective immunization, while immunization with irradiated Meth A tumor cells required CD4 and CD8 cells but no macrophages (3).

For priming one million unirradiated, live E.G7-secreting gp96-Ig were inoculated s.c. This dose is sufficient to establish tumors that grow to a mean diameter of about 8 mm, subsequently shrink, and are rejected. Tumor rejection is blocked in mice treated with the anti-CD8 Ab 2.43, either 2 days before (Fig. 3A) or up to 3 days after tumor inoculation (not shown). The anti-CD4 Ab GK1.5 had no effect on tumor rejection (Fig. 3A) regardless of time of injection, even though it completely depleted CD4 cells for >14 days (data not shown). CD4-deficient mice were able to reject E.G7-gp96-Ig (Fig. 3B), supporting the importance of CD8 cells. E.G7 not secreting gp96-Ig forms tumors in untreated and immune-depleted mice. Carrageenan, known to inactivate macrophages *in vivo* (34), had no effect on tumor rejection. However, because the effect of Carrageenan is difficult to assess, these ex-

periments cannot rule out a role for APCs in the generation of CD8 CTL.

To study the effector phase of tumor rejection, mice were immunized twice at 14-day intervals with live E.G7-gp96-Ig. Eleven days later (day 25), immune cells were depleted, and after 3 days the mice were challenged with untransfected E.G7. Only CD8 cells are required in the effector phase; depletion of CD4 cells or Carrageenan inactivation of macrophages had no influence on E.G7 rejection in the effector phase (Fig. 3C).

Discussion

Deletion of the endoplasmic retention signal of gp96 and replacement with the Fc portion of IgG1 readily results in the secretion of gp96-Ig, which appears to be dimerized through the IgG1 H chain. E.G7-secreting gp96 is able to provide long-lasting specific immunity, suggesting that it chaperones tumor peptides. In contrast, irradiated or mock-transfected E.G7 are not able to provide protective immunity. *Corynebacterium parvum* also failed to serve as adjuvant for E.G7 immunization (37). Secreted gp96-Ig provides immunologic specificity for both the surrogate Ag OVA and other EL4 Ags, but does not cross-immunize to LLC-derived tumor Ags.

The data are consistent with the explanation that peptides associated with secreted gp96-Ig are transferred to and presented by class I MHC and stimulate a tumor-specific CD8⁺ CTL response

causing tumor rejection. The CD8 response appears to be independent of CD4 help and does not require macrophages. Whether the cellular requirements are due to gp96-Ig dimerization is not known.

It is instructive to compare the mechanisms of immunization by purified tumor-derived gp96 and by tumor-secreted gp96-Ig. Udono et al. (32), using gp96 purified from Meth A tumor cells for immunization, reported a requirement for CD8 cells and macrophages in the priming phase and a requirement for CD4 and CD8 cells as well as macrophages in the effector phase of tumor rejection of Meth A tumors. Immunization with irradiated Meth A tumors required CD4 cells in the priming phase, and both CD4 and CD8 cells in the effector phase. Irradiated EG7 do not produce immunity against subsequent challenge. The dramatic effect of tumor-secreted gp96-Ig is entirely dependent on CD8 cells without CD4 help. CD8 cells are required in the priming and effector phase of the CTL response to the tumor. Macrophages appear not to be needed. The role of dendritic cells or other APCs in the presentation of gp96-chaperoned peptides to CD8 cells is not known, but remains a possibility. It is also possible that gp96-Ig-secreting EG7 stimulate CD8 cells directly.

Regardless of the underlying mechanism, tumor-secreted gp96-Ig is a powerful vaccine for some tumors. The precise mechanism CD8 CTL activation by tumor-secreted gp96-Ig and its action in different tumors remains to be determined.

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Small Cell Lung Carcinomas Express Shared and Private Tumor Antigens Presented by HLA-A1 or HLA-A2¹

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ABSTRACT

Tumor-derived peptides presented by MHC class I molecules are targets for tumor rejection by CD8⁺ CTLs. MHC-restricted CD8⁺ CTLs are required also for the identification and characterization of tumor antigens that will be useful for immune therapy. For many human solid tumors, however, tumor antigens remain undefined because of the difficulty of generating MHC-restricted, tumor-specific CTLs required for their analysis. CD8⁺ CTL responses are modulated by CD4⁺ helper T cells and by antigen-presenting cells. In this study, highly purified CD8⁺ T cells were mixed with tumor cells in primary cultures in the absence of any other cells to reduce the complexity of CTL generation. Tumor cells were transfected with HLA-A1 or HLA-A2 and used to stimulate partly matched HLA-A1- or HLA-A2-positive CD8⁺ T cells. Partial MHC class I matching of tumor and CD8⁺ T cells and omission of other cells in primary culture was highly effective in generating MHC class I-restricted CTL to poorly immunogenic small cell lung carcinomas (SCLCs). Cytotoxicity was further enhanced by cotransfection of tumor cells with B7.1 (CD80). ICAM-1 (CD54) was not as effective as costimulation. SCLC cells presented tumor-specific peptides with HLA-A1 and HLA-A2 and were lysed by A1- or A2-restricted CD8⁺ CTLs. A1- and A2-restricted CD8⁺ CTLs detected shared tumor antigens on unrelated SCLC tumor lines in addition to private antigens. The use of direct antigen presentation by MHC class I-transfected tumors to MHC class I-matched CD8⁺ T cells is an effective way to generate MHC class I-restricted CTLs toward poorly immunogenic tumors *in vitro*, permitting the molecular identification of their tumor antigens.

INTRODUCTION

Tumor progression *in vivo* requires evasion of an effective immune response. Tumors have evolved various strategies for immune avoidance (1, 2). Immunosuppressive tumors, such as gliomas, produce or stimulate production of factors including transforming growth factor β (3) and IL-3-10 (4) that suppress or deviate a cellular cytotoxic immune response to the tumor. Immunogenic tumors represented by melanomas and renal cell carcinomas provoke an immune response that, however, does not proceed to tumor cell lysis, in part because of the expression of death ligands, such as Fas-L (5) or other death ligands (6, 7). Another large group of tumors that includes lung carcinomas avoid an immune response by reducing their immunogenicity through the down-regulation of MHC class I molecules (8-10) or by blockade of antigen processing or peptide transport for MHC class I loading (9-11). Individual tumors may use several components of these strategies concurrently.

Costimulatory molecules for T-cell activation, such as B7.1 and B7.2, are highly expressed on activated APCs. Transfection of B7.1 or B7.2 and expression in tumor cells has been shown to increase their

immunogenicity and mediate their *in vivo* rejection in experimental animals (12, 13) as well as facilitate CTL generation against melanoma *in vitro* (14, 15).

A number of tumor antigens have been identified for immunogenic tumors such as melanoma (16-23), but little is known about tumor antigens in low or nonimmunogenic tumors, such as lung tumors, and their MHC presentation (24-26). To begin to identify tumor antigens on this large group of human tumors, it is necessary to devise methods to render these tumors antigenic and to define MHC restriction elements for tumor peptides. MHC restriction of CTL responses to tumor antigens in humans has been studied extensively in melanoma, and many of the identified tumor antigens can be presented by HLA-A2 and HLA-A1 (17-21, 27-30). The frequency of HLA haplotype expression is thought to reflect their use for directing T-cell responses to viral and tumor-derived peptide recognition (31). HLA-A1 and HLA-A2, expressed with high frequency in the Caucasian population, therefore, were chosen in this study to survey the presence of tumor antigens on SCLC cells and their restriction by HLA-A1 or HLA-A2.

CD8⁺ CTL responses to tumors are thought to be important for tumor rejection. MHC class I-restricted, peptide-specific CD8⁺ CTLs are also required for the discovery and analysis of tumor antigens (17-19, 21-23, 29). However, CD8⁺ CTL activation is a complex process, usually involving at least three cell types in addition to the tumor cells, *i.e.*, APC, CD4⁺ helper T cells, and CD8⁺ CTLp (32-34). The role of the APC is to present peptide via MHC classes I and II and to provide appropriate costimuli such as B7 (32, 35, 36), whereas CD4⁺ helper T cells have recently been shown to induce B7 expression on APCs through CD40-Ligand/CD40 interaction (37-40). CD4⁺ helper T cells, in addition, produce IL-2 and other cytokines that facilitate CD8⁺ CTL expansion. The complexity of CD8⁺ CTL induction is further compounded by the potential of APCs to induce tumor-specific tolerance (41) and of CD4⁺ T cells to be suppressive (42, 43). We therefore sought to define a less complex system for the generation of MHC class I-restricted CD8⁺ CTLs specific for non- or low immunogenic tumors. As described in this report using transfected SCLC cells and purified CD8⁺ T cells without additional cells in primary stimulation, we were able to generate SCLC-specific CTLs and to demonstrate the presence of shared antigens that can be restricted by HLA-A1 and HLA-A2.

MATERIALS AND METHODS

Cell Lines, cDNAs, and Transfection

Human SCLC cell lines (SCLC#2 and SCLC#7) and human lung adenocarcinoma cell lines (AD#100 and AD#101) were established as described (44). The human melanoma cell line, MEL#113, was obtained from Dr. Levy of the Department of Microbiology and Immunology. K562 was provided by American Type Culture Collection. All cells were cultured in IMDMEM medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.). Lung carcinoma cell lines and melanoma cell lines were maintained as monolayer cultures and passaged by short trypsinization with 0.05% trypsin plus EDTA (Life Technologies, Inc.) as required.

HLA-A0101 (A1) and HLA-A0201 (A2) cDNAs were kindly provided by Dr. Peter Parham (Stanford University, Palo Alto, CA). They were cloned into the eukaryotic expression vector, pBCMGSNeo (45), or a new double expres-

Received 4/15/99; accepted 7/21/99.

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¹ This work was supported by NIH Grants CA39201 and CA57904.

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³ The abbreviations used are: IL, interleukin; APC, antigen-presenting cell; SCLC, small cell lung carcinoma; CTLp, CTL precursor; ICAM, intercellular adhesion molecule; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; NK, natural killer.

sion vector, B45NeoCM, constructed from B45Neo (46) to express two genes by two different promoters, the cytomegalovirus promoter and the mouse metallothionein promoter.⁴ Human B7.1 cDNA was generated by reverse transcription-PCR with primers that amplified the cDNA between the ATG codon of the leader peptide and the termination codon (47). The PCR product was cloned into the eukaryotic expression vector, pBGMGHIS (48–50), or the double expression vector, B45NeoCM. Human ICAM-1 was kindly provided by Dr. Lewis L. Lanier (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) in a hygromycin-selectable expression vector, SRa296-hygro. The cDNAs were used to transfect SCLC#2, SCLC#7, AD#100, AD#101, and MEL#113 by Lipofectin (Life Technologies, Inc.). Transfected cells were selected with 1 mg/ml of G418 (Life Technologies, Inc.) for pBGMGHIS or B45NeoCM, with 5–10 mM of L-Histidinol (Sigma Chemical Co., St. Louis, MO) for pBGMGHIS, or with 800 µg/ml of hygromycin B (Calbiochem-Novabiochem Corporation, La Jolla, CA) for SRa296-hygro for at least 2 weeks. Cells highly expressing HLA-A1, HLA-A2, B7, or ICAM-1 were sorted on a FACStar flow cytometer (Becton Dickinson, Mountainview, CA) and expanded to cell lines.

Antibodies

Mouse anti-human CD8 monoclonal antibody OKT8 (IgG2a) was purified from hybridoma supernatants, followed by protein G affinity chromatography. Monoclonal antibodies against HLA-A1, A36 and HLA-A2, A24 (IgM) were purchased from One Lambda (Canoga Park, CA) and used diluted at optimal concentration. Phycoerythrin-labeled anti-BB1/B7 antibody (B7-PE) was purchased from Becton Dickinson. Biotin-labeled anti-human CD54 (ICAM-1-Biotin) was purchased from Southern Biotechnology Associates (Birmingham, AL). Secondary antibodies for fluorescence-activated cell sorting included FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgM (Boehringer Mannheim, Indianapolis, IN) at a dilution of 1:40 and streptavidin-PerCP (Becton Dickinson) at a dilution of 1:10. Microbeads conjugated with goat anti-mouse IgG (Miltenyi Biotec, Sunnyvale, CA) were used as secondary reagent for magnetic cell sorting.

Immunofluorescence Phenotyping of MHC Class I Molecules, B7.1 and ICAM-1

For surface expression of HLA-A1 and HLA-A2, 5×10^5 tumor cells were incubated with optimal concentrations of anti-HLA-A1, A36 or anti-HLA-A2, A24 monoclonal antibody or isotype-matched control antibody for 30 min at 4°C, followed by incubation with FITC-conjugated goat anti-mouse IgM for 15 min at 4°C. For surface expression of B7.1, 5×10^5 tumor cells were incubated with optimal concentrations of B7-PE or isotype-matched control antibody for 30 min at 4°C. For surface expression of ICAM-1, 5×10^5 tumor cells were incubated with optimal concentrations of ICAM-1-Biotin or isotype-matched control antibody for 30 min at 4°C, followed by incubation with streptavidin-PerCP for 15 min at 4°C. The cells were washed, resuspended in PBA [PBS supplemented with 0.5% BSA (Sigma, St. Louis, MO) and 5 mM EDTA (Sigma), pH 7.2], and analyzed by a Becton Dickinson FACScan flow cytometer. For double or triple staining of HLA-A1 or HLA-A2, B7, and ICAM-1, cells were stained in the order of HLA-A1 or HLA-A2, B7, and ICAM-1.

HLA Typing

HLA typing of tumor cells or of PBLs from normal healthy volunteers was done in the Division of Transplantation Surgery, University of Miami (51).

Isolation of CD8⁺ T Cells

PBMCs were obtained from peripheral blood from two normal healthy volunteers. Heparinized samples were diluted with PBS, overlaid onto Ficoll-

Paque (Pharmacia Biotech, Uppsala, Sweden), and centrifuged at 1400 rpm for 30 min. The lymphocyte-rich layer at the interface was then washed three times with PBS and resuspended in PBS to stain for isolation of CD8⁺ T cells. CD8⁺ T cells were obtained by magnetic cell sorting (52). Briefly, PBMCs were labeled with mouse anti-human CD8 monoclonal antibody OKT8 for 15 min at 4°C, followed by incubation with microbead-conjugated goat anti-mouse IgG for 15 min at 4°C in PBA. Cells were immediately applied to a MACS separation column type AS (Miltenyi Biotec), which was placed in the separator. The quality of the separation was subsequently determined by flow cytometry after labeling with FITC-conjugated goat anti-mouse IgG. It is important to obtain highly purified CD8⁺ T cells and eliminate dimly CD8⁺-positive NK cells by using low concentrations of anti-CD8 antibody (1:4000) to avoid overgrowth of cultures by NK-type cells.

Establishment of CTL Lines in Microcultures

Various numbers (10^3 , 3×10^3 , and 10^4) of highly purified, NK (CD8⁺dim)-depleted, CD8⁺ T cells were mixed in 96 round-bottomed microwells (Costar, Cambridge, MA) with 300 tumor cells, irradiated with 12,000 rads from a Cobalt source as stimulator cells in 160 µl of complete medium prepared from IMDMEM medium supplemented with 10% FCS, 50 µM of β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), and 50 µg/ml of gentamicin (Life Technologies, Inc.), and incubated at 37°C in 5% CO₂. IL-2 (Hoffman-La Roche, Nutley, NJ), at a final concentration of 25 IU/ml, and IL-4 (Genzyme, Cambridge, MA), at a final concentration of 50 IU/ml, were added on day 5 (final volume, 200 µl). The microcultures were restimulated on days 8 and 15 by replacing 100 µl of fresh complete medium containing IL-2 (50 IU/ml), IL-4 (100 IU/ml), 300 irradiated tumor cells, and 10^5 allogeneic or autologous PBLs, irradiated with 4000 rads, as feeder cells. Proliferating cells were transferred into 96 flat-bottomed microwells (Costar) on days 14 to 19. When the number of the cells reached approximately 3×10^5 /well, they were transferred into 24-well plates (Costar). On day 20, cytotoxic activity of the cells was tested against wild-type, HLA-matched, and HLA-mismatched tumor cells and K562 by 4-h Cr-release assays. Long-term culture of CTLs was achieved by repeating restimulation as above.

CTL Cloning

Expanded CTL lines were seeded at 3, 1, and 0.3 cells/well in 200 µl of complete medium containing IL-2 (25 IU/ml), IL-4 (50 IU/ml), 300 irradiated tumor cells, and 10^5 irradiated allogeneic PBMCs in 96 round-bottomed microwells. The microcultures were restimulated on days 8 and 15 by replacing 100 µl of fresh complete medium containing IL-2 (50 IU/ml), IL-4 (100 IU/ml), 300 irradiated tumor cells, or 10^5 irradiated allogeneic PBMCs. In the third week, cytotoxic activity of the cells was tested against wild-type, HLA-matched, and HLA-mismatched SCLC cells and K562 by Cr-release assays.

Cytotoxicity Assays

Target tumor cells were labeled with Na₂⁵¹CrO₄, and 100-µl aliquots containing 5×10^3 labeled tumor cells were distributed into 96 round-bottomed microwells containing 100 µl of medium alone or 5×10^4 CTLs in medium to result in an E:T ratio of 10:1. To analyze MHC class I specificity of tumor-specific CTLs, at least 1.5×10^5 effector cells were required. The plates were then centrifuged for 30 s at $200 \times g$ and incubated for 4 h at 37°C in 5% CO₂. The plates were centrifuged again for 5 min at $200 \times g$, and 100 µl of supernatant were collected and counted in a gamma counter (LKB-Wallac RiaGamma; Wallac Oy, Finland). The percentage of ⁵¹Cr-specific release was calculated as follows:

$$\% \text{ specific release} = \frac{ER - SR}{MR - SR} \times 100$$

where *ER* was the observed experimental ⁵¹Cr release, *SR* was the spontaneous release measured by incubation of 5×10^3 labeled cells in 200 µl of medium alone, and *MR* was the maximum release obtained by adding 100 µl of 2%

⁴ Unpublished data.

Triton X-100 (Sigma) to 100 μ l of the target cells. The spontaneous release of most target cells ranged between 10 and 15% of the maximum release.

Evaluation of Results and CTLp Frequency

Specific Lysis. Values of specific lysis exceeding three times the SD of the spontaneous release were considered significant lysis in the whole study. For example, if spontaneous lysis was 10% with 3% SD, then specific lysis by CTL had to be >19% to be considered as significant. In most experiments, specific lysis exceeded spontaneous lysis by 10% or more.

MHC Restriction of Lysis and Specificity. Differences in lysis observed with different tumor targets, *i.e.*, wild-type, A1-, or A2-transfected, were evaluated using the Student *t* test. We defined a 30% difference in the specific lysis of different targets as significant. For example, A1-restricted CTLs are expected to lyse A1-transfected targets at least 1.3 times better than the A2 or wild-type targets. Experiments were set up in triplicate, and a significant difference in the lysis ($P < 0.05$) of different target cells was observed in most experiments.

Allogeneic and Promiscuous CTLs Are Defined as CTLs That Lyse Wild-Type and Transfected SCLC Cells Equally Well and at Least 10% Above Spontaneous Release. Nonspecific or noncytotoxic cells are those that lyse only K562 or none of the target cells used in the cytotoxicity assay.

Costimulatory Molecules. To compare the costimulatory efficacy of MHC class I molecules with B7.1 and ICAM-1, 10^4 CD8⁺ T cells were stimulated and restimulated with 300 irradiated wild-type or transfected SCLC cells. More than 150 wells for each condition were set up in 20 experiments with CD8⁺ T cells from A1- and A2-positive volunteers. The significance of the difference between using different costimulators was evaluated on the basis of the Student *t* test.

CTLp Analysis. For CTLp analysis, the percentage of negative wells in cytotoxicity assays was plotted on a logarithmic scale against the number of responder CD8⁺ T cells plated at the beginning of the experiment on a linear scale (53, 54). Linear regression analysis of number of responder cells/well against log percentage of negative wells was then performed, and the frequency of CTLp was determined on the basis of the Poisson distribution (53).

RESULTS

Partial MHC Class I Matching of Stimulator Tumor Cells and Responder CD8⁺ T Cells Generates Tumor-specific, MHC Class I-restricted CTLs. HLA-A1 and HLA-A2 are frequently expressed MHC alleles in the Caucasian population and are effective in presenting tumor peptides to CD8⁺ T cells. A1 and A2, therefore, are ideal molecules to test the presentation of tumor peptides in tumors with as yet undefined tumor antigens and restriction elements. To affect optimal CTL stimulation by tumor cells, high expression of matched HLA and costimulatory molecules may be necessary, especially for poorly immunogenic tumors such as lung tumors.

SCLC tumor cells frequently show low expression of MHC class I molecules. This was observed also for the cell lines used in this study; HLA-A2 was detected in SCLC#7 at a very low level, whereas HLA-A1 was not detected in any of the tumor cell lines used in this study. Expression of high levels of HLA-A1 or HLA-A2 on tumor cells was achieved by transfection of the tumor cells with HLA-A1 or HLA-A2 cDNA.

Singly transfected tumor cells expressing HLA-A1 or HLA-A2 or B7.1 (CD80) or ICAM-1 (CD54), double transfectants expressing HLA-A1 and B7.1, HLA-A2 and B7.1, HLA-A1 and ICAM-1, or HLA-A2 and ICAM-1, and triple transfectants expressing HLA-A1, B7.1, and ICAM-1, or HLA-A2, B7.1, and ICAM-1 were prepared from the two SCLC cell lines, SCLC#2 and SCLC#7. The transfectants were used for both purposes, as stimulator cells for mixed CD8⁺ T-cell tumor cell cultures and as target cells for cytotoxicity assays. Fig. 1 shows expression levels of the transfected cDNAs HLA-A1, HLA-A2, B7.1, and ICAM-1 on SCLC#2 and SCLC#7 by flow cytometry. Neither wild-type SCLC#2 nor SCLC#7 expresses HLA-

A1. SCLC#7 is HLA-A2 positive by HLA typing, but its expression level of HLA-A2 was much lower than that of HLA-A2 on PBLs of a healthy volunteer (not shown). After transfection of tumor cells with HLA-A1 or HLA-A2, their expression levels increased to the levels found on PBLs. Wild-type SCLC#2 expressed very low levels of ICAM-1 but no B7.1, whereas wild-type SCLC#7 expressed neither B7.1 nor ICAM-1. However, after transfection with B7.1 or ICAM-1, their expression levels increased, as shown in Fig. 1. Double and triple transfectants were made by transfection and sorting for the positive population and then transfecting with the next cDNA. Almost 100% of the cells expressed HLA-A1 or HLA-A2, B7.1, and/or ICAM-1 in each double or triple transfectant, as determined by two- and three-color flow cytometric analysis (not shown). HLA-A2 expression levels were very similar on single, double, or triple transfectants of SCLC#7 (Fig. 1). However, HLA-A1 expression levels on the triple transfectant SCLC#2-HLA-A1-B7-ICAM-1 was somewhat lower than on the other SCLC#2 transfectants, despite selection pressure. We were unable to generate stable HLA-A1 expressing double or triple transfectants with SCLC#7. After initial HLA-A1 expression, the cells invariably lost their expression within two to three weeks after transfection, even when sorted for high-level HLA-A1 expression and maintained under selection pressure. Similarly, HLA-A2 expression on double and triple transfectants of SCLC#2 was unstable for reasons that are not clear. Therefore, the series of SCLC#2-HLA-A1 transfectants were used as stimulators to generate HLA-A1-restricted CTLs and the series of SCLC#7-HLA-A2 transfectants as stimulators to generate HLA-A2-restricted CTLs.

Highly purified, HLA-A1-positive CD8⁺ T cells were stimulated with irradiated wild-type SCLC#2, SCLC#2-B7, SCLC#2-HLA-A1, or SCLC#2-HLA-A1-B7. During primary culture, only tumor cells and highly purified CD8⁺ T cells were present. The presence of other cells including CD8⁺dim during primary culture diminished tumor specificity and HLA-A1 restriction while increasing nonspecific proliferation. After 7 and 14 days, the proliferating CD8⁺ T cells were restimulated with the same transfectants used originally, together with irradiated autologous or allogeneic PBLs. During secondary and tertiary stimulation, feeder cells were required to enhance proliferation of tumor-specific CTLs. Cytotoxic activity toward several target cells was tested in triplicate on the 20th day by Cr-release assay at an E:T ratio of 10:1. Wells stimulated with SCLC#2-HLA-A1 or SCLC#2-HLA-A1-B7 proliferated sufficiently to generate enough cells for Cr-release assays, but wells containing wild-type SCLC#2 as stimulators frequently did not have enough cells for assay. To assess the specificity of the CTLs generated, their cytotoxicity was assessed routinely with four targets: against the HLA-A1-transfected, matched tumor cells used as stimulator; against mock-transfected, wild-type tumors, against the mismatched HLA-A2 transfectants; and against the NK target K562.

The stimulation of A1-matched CD8⁺ T cells with HLA-A1-transfected SCLCs is expected to produce HLA-A1-restricted CTLs, if HLA-A1 can serve as a restricting element for tumor peptides and if CD8⁺ CTLp with appropriate specificity are present. Because allogeneic responses to unmatched HLA alleles will also occur in this system, it is critical to distinguish A1 (or A2 when applicable) restricted lysis from allogeneic or other CTL responses. This is particularly important because the microcultures may have several clonal specificities in each well, including the desired A1 (or A2)-restricted CTLs, allogeneic CTLs, or promiscuous CTLs. The assay system is designed to distinguish between HLA-A1 (or A2)-restricted CTL responses and other allogeneic or nonspecific responses by analyzing CTL activity against the four targets as noted above. Three types of specificities were observed in the analysis of CTL microcultures and were defined as follows. HLA-A1 (or A2)-restricted CTLs

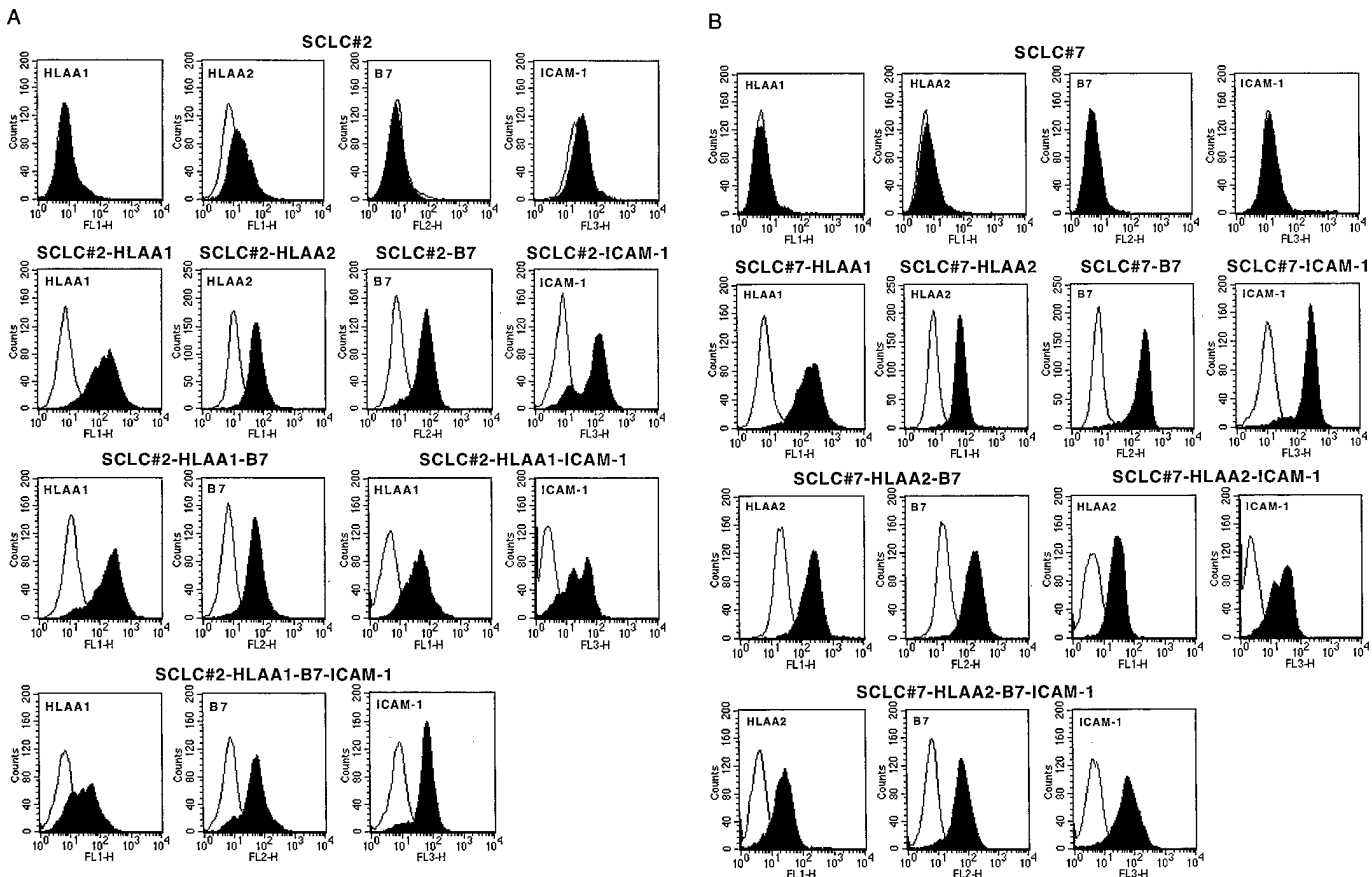


Fig. 1. Analysis of wild-type and transfected SCLC#2 (A) and SCLC#7 (B) by flow cytometry. Tumor cells were stained with anti-HLA-A1 or anti-HLA-A2 antibody, followed by FITC-conjugated goat anti-mouse IgM and anti-B7-PE or by anti-ICAM-1-Biotin and streptavidin-PerCP and analyzed by flow cytometry. *Black histograms*, samples stained with specific antibodies; *white histograms*, samples stained with isotype immunoglobulin control. The specificity of the antibody is to the antigen noted inside the box; the transfectant used is noted above the box.

are required to lyse HLA-matched SCLC cells at least 1.3 times better than wild-type SCLC cells or HLA-mismatched SCLC cells (Fig. 2, *arrows*). Allogeneic or promiscuous CTLs are defined as CTLs that lyse wild-type and HLA-transfected SCLC cells equally well. Non-

specific or noncytotoxic cells are those that either lyse only K562 or none of the target cells used in the cytotoxicity assay. In Fig. 2, the results of a representative experiment are shown comparing the effects of stimulator cell transfection with A1 and B7 on the specificity of the

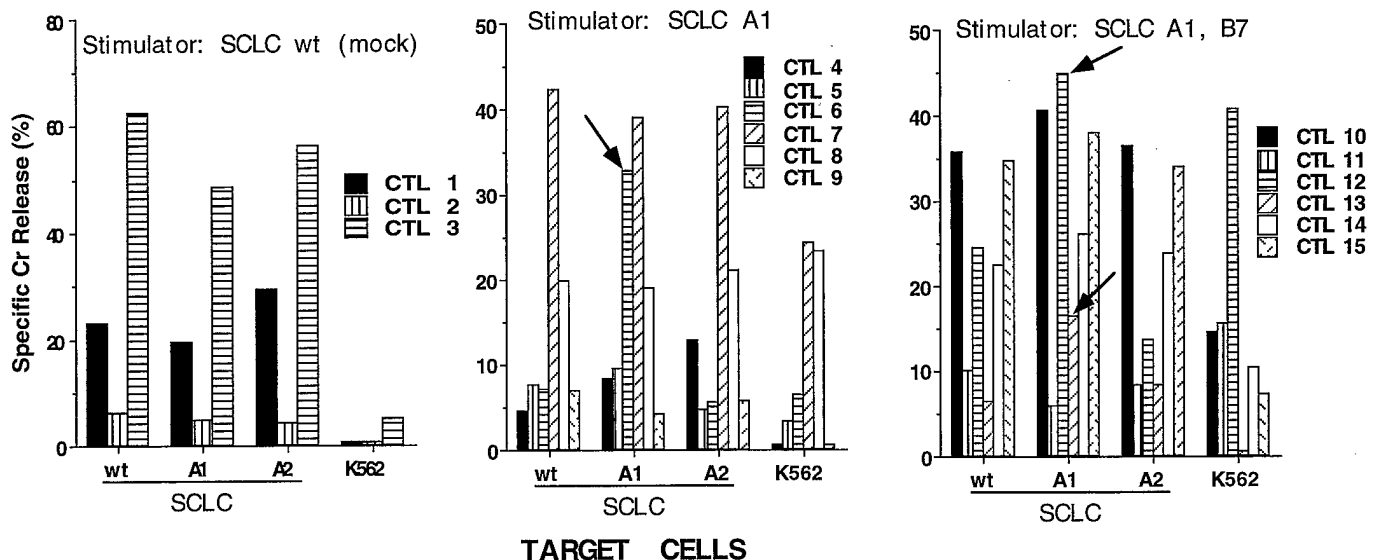


Fig. 2. Specificity of HLA-A1-positive CD8 CTLs after stimulation with mock-transfected or HLA-A1/B7.1-transfected tumor cells. Six wells were used for each tumor stimulator, SCLC#2 (mock), SCLC#2-A1, and SCLC#2-A1-B7.1 for stimulation of CD8 T cells as described. Cytotoxicity was examined on day 20 of culture. The E:T ratio was 10:1 and in 4-h assays against the four targets indicated at the *abscissa* of the graph, SCLC#2 (mock), SCLC#2-A1, SCLC#2-A2, and K562. The specific cytotoxicity of individual wells is plotted. *Arrows*, wells that fulfill criteria for MHC restriction by A1, defined as 1.3-fold or higher lysis in comparison with mock-transfected or A2-transfected SCLCs.

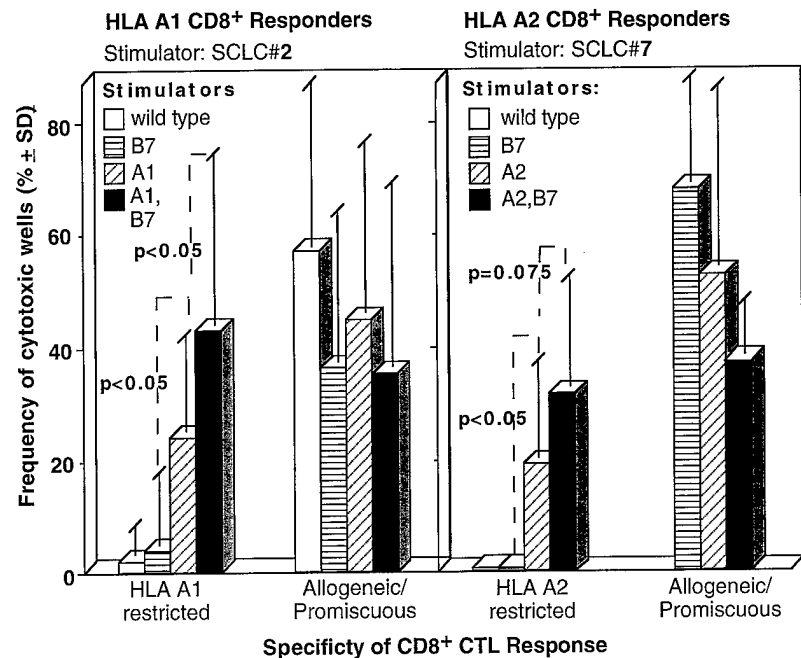


Fig. 3. Frequency of specificity of CD8 CTLs after stimulation with wild-type, B7.1-transfected, class I-transfected, or double transfected SCLCs. For stimulation in the *left panel*, SCLC#2 and HLA-A1 was used; in the *right panel*, SCLC#7 and HLA-A2. The data from 20 experiments are calculated, and the frequency of wells containing MHC-restricted CTLs *versus* nonrestricted CTLs is shown in dependence of the transfection of the stimulator tumor cell. Statistical significance is indicated by Ps. Culture conditions were as described in "Materials and Methods."

CD8⁺ CTL response. When wild-type SCLCs were used as stimulators, only three of six wells proliferated sufficiently to allow analysis. Of the three CTL microcultures generated with wild-type SCLCs, none fulfilled the criteria for A1 restriction (Fig. 2, *left panel*). A1 transfection increased CTL expansion during culture, and one of six wells (Fig. 2, *middle panel*, arrow) lysed A1-transfected targets with higher specificity than other targets. Double transfection of SCLCs with A1 and B7 further increased cytotoxicity and frequency of A1 restriction (arrows).

The cumulative results of 480 wells analyzed against four targets in 20 similar experiments using A1 or A2 as restriction element for SCLCs and their statistical analyses are summarized in Fig. 3. In the absence of HLA transfection, wild-type and B7.1-transfected tumor cells produced only allogeneic responses, lysing untransfected and A1- or A2-transfected SCLCs to a similar extent. HLA-A1-transfected stimulators increased the frequency of HLA-A1-restricted CTLs significantly ($P < 0.05$). B7 transfection of SCLCs, together with HLA, had two effects. It increased the frequency and level of cytotoxicity in all wells, and significantly, it increased the frequency of A1-restricted CTLs ($P < 0.05$). Similarly, SCLC#7-HLA-A2 stimulation helped to generate HLA-A2-restricted CTLs ($P < 0.05$ *versus* SCLC#7 and SCLC#7-B7). Double transfection of SCLC#7-HLA-A2-B7 increased the frequency of HLA-A2-restricted CTLs, compared with HLA-A2 transfection alone, although the value was not significant ($P = 0.075$).

The criteria used to define MHC restriction used are conservative and are likely to underestimate the true frequency. For instance, CTLs that lyse wild-type and HLA-A1-transfected SCLCs equally well may contain both allogeneic CTLs and A1-restricted CTLs. They would, however, not be counted as A1 restricted by the definition used because the allogeneic specificity cannot be distinguished from A1 restriction without cloning.

To confirm specificity and HLA-A1 or HLA-A2 restriction of CTLs, single-cell cloning experiments using limiting dilution techniques were carried out. Expansion of clones from 3, 1, or 0.3 cells/well required 3 weeks of culture with periodic restimulation. All clones were analyzed for specificity on a panel of target cells as above. An example of one cloning experiment is shown in Fig. 4A. Eight clones generated by limiting dilution and culture for 3 weeks

were compared with the specificity of the uncloned parent A1-specific CTL line containing also lytic activity for K562. During the cloning period, cytotoxic activity decreased, but MHC class I-restricted specificity was increased. Thus, after cloning, few allogeneic or promiscuous CTL clones were present, despite their presence at the initiation of the cloning procedure. A large fraction of clones were HLA-A1-restricted CTLs with high specificity for HLA-A1 and variable cross-reactivity for HLA-A2 but low activity for K562 or wild-type SCLCs (Fig. 4B). The cumulative data of 48 clones generated from >10 A1-restricted lines indicate that an equal number of clones retained A1 specificity or lost their activity for any of the target cells tested, respectively. Only very few clones retained allogeneic specificity.

The data suggest that continuous culture and restimulation of tumor-specific CTLs with HLA-matched SCLC cells for longer periods of time increased the specificity and frequency of MHC class I-restricted CTLs over that of allogeneic or promiscuous CTLs (Fig. 4). However, prolonged culture of CTLs also resulted in a gradual decrease of cytotoxicity and a decreased rate of proliferation (not shown).

Shared and Private HLA-A1- and HLA-A2-restricted Tumor Antigens Expressed by Two Unrelated SCLC Lines. The development of immunological tumor therapy is facilitated by the presence of shared tumor antigens expressed by the majority of tumors of the same type obtained from different patients. It was therefore important to determine whether A1-restricted CTLs could lyse unrelated SCLC lines after transfection with A1. Two unrelated A1- and A2-transfected SCLC lines were used as targets. As additional controls, non-SCLC and melanoma cells were transfected with HLA-A1 and used as targets. Thirty-six of 47 individual, A1-restricted CTL lines (76.6%) generated with SCLC#2-HLA-A1 also lysed the unrelated SCLC#7-HLA-A1, indicating the presence of one or more shared peptide epitopes presented by A1 (Table 1). Eleven of the 47 CTL lines lysed only the A1-transfected SCLC#2 line that was used for CTL generation but not SCLC#2, suggesting that these CTLs recognized A1-restricted, private antigens not expressed by unrelated SCLCs. The reciprocal experiments showed comparable results in that 29 of 40 CTL lines (72.5%) lysed the two unrelated A1-transfected tumors. Fewer A2-restricted CTL lines were tested, but three of four

Fig. 4. Frequency of specificity of CTL clones generated by limiting dilution analysis. A, one HLA-A1-restricted CTL line was diluted and seeded at 3, 1, and 0.3 cells/well, together with irradiated SCLC#2-HLA-A1 and irradiated allogeneic PBMCs in 96 round-bottomed microwells. The microcultures were restimulated on days 8 and 15. On day 20, all clones that proliferated were analyzed for specificity on the panel of target cells as indicated by Cr-release assays. B, cumulative frequency of HLA-A1-restricted CTLs, allogeneic CTLs, and noncytotoxic cells obtained from the analysis of 48 clones obtained in a similar way as in A.

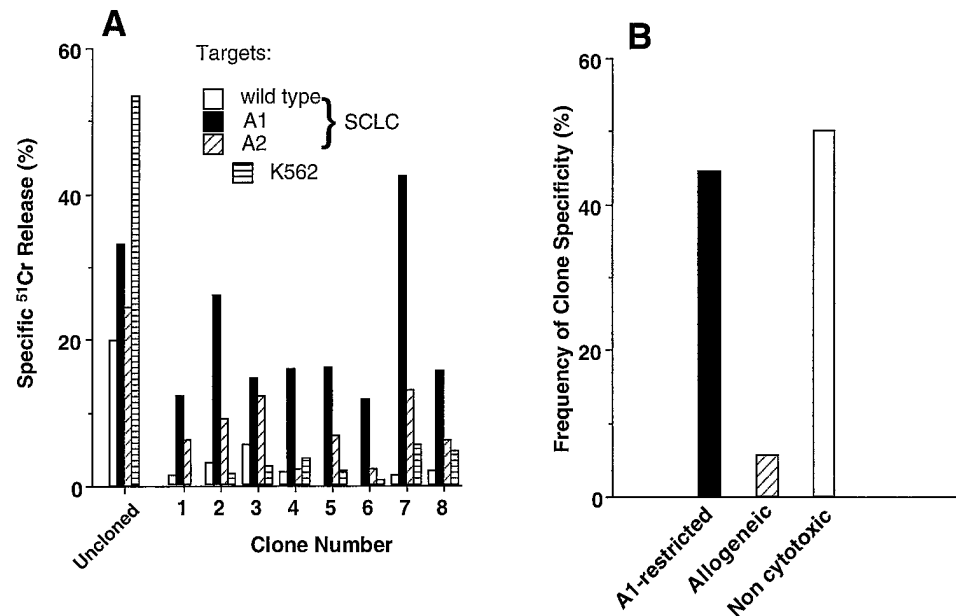


Table 1 Shared antigens presented by HLA-A1 in the two unrelated lines, SCLC#2 and SCLC#7

CTLs were generated from purified CD8 cells, as described in the text, using the stimulator cells indicated. Individual CTL lines were tested against either A1-transfected SCLC line in Cr-release assays for 4 h. Lysis was scored positive when the unrelated target showed 80% or more of the lysis compared with lysis of the stimulator cell.

Stimulator cells	Target cells lysed/CTL lines tested	
	SCLC#2-A1	SCLC#7-A1
SCLC#2-A1	47/47	36/47
SCLC#7-A1	29/40	40/40

A2-restricted, CTLs tested detected shared A2-restricted antigens in an unrelated line (Table 2). In contrast, only one of seven A1-restricted SCLC-specific CTL lines lysed other A1-transfected tumor types such as non-SCLCs and a melanoma (Table 3). Apparently, the majority of SCLC-specific CTLs recognize antigens not found on other tumors. These data also exclude the possibility that HLA-A1- or HLA-A2-restricted CTLs are specific for peptides derived from the bovine papilloma virus expression vector that was used for the expression of HLA-A1 and other cDNAs in all tumor lines used.

MAGE 1 and MAGE 3 is expressed by several tumors, including melanoma and lung tumors, and their peptides are presented by A1 (18, 29). However the HLA-A1-restricted CTL line in Table 3 that lysed several A1-transfected tumors did not lyse MAGE-peptide pulsed A1-positive B cells (data not shown).

Role of B7.1 and ICAM-1 in CTL Generation. ICAM-1 interaction with LFA-1 is thought to deliver an important first signal for T-cell activation, rendering T cells permissive to subsequent signaling by other molecules (55). Because SCLC lines express very low to undetectable levels of ICAM-1, we determined the effect of ICAM-1 transfection on CTL generation of stimulator tumor cells together with B7.1 transfection (Fig. 5). ICAM-1 cotransfection with MHC class I had only a modest effect on the frequency of MHC class I-restricted CTL generation when compared with B7.1 plus MHC class I. When ICAM-1 was cotransfected in triple transfectants with MHC class I and B7.1, it reduced the frequency of CTL generation in comparison to the double transfectants of B7.1 and MHC class I, although the effect was not statistically significant. The effect of ICAM-1 expression, although detectable, did not seem limiting or essential in this system. In contrast, the costimulatory effect of B7.1 is significant if

the signals derived from B7.1 are presented by the same cell as MHC class I (in *cis*). In the *trans* situation, when SCLC#2-HLA-A1 (or -A2) and SCLC#2-B7 were mixed and used as stimulators, the frequency of CTL generation was reduced in comparison with the effect of double transfectants of B7.1 and HLA-A1 (or HLA-A2) on the same cell ($P < 0.01$).

Analysis of CTLp Frequency in CD8⁺ T Cells of Normal Healthy Volunteer's PBMCs. MHC class I-restricted CTLp frequencies were measured by stimulation of T cells with tumors coexpressing HLA-A1 or HLA-A2 with B7.1. Poisson analysis of the number of responder cells generating specific CTLs (Fig. 6; Ref. 53) showed CD8⁺ CTLp frequencies of 0.45×10^{-4} and 0.38×10^{-4} in the HLA-A1 and HLA-A2 volunteers, respectively.

DISCUSSION

Several tumor antigens have been identified in human tumors (16–23), allowing their evaluation in immunotherapy. However, most human tumors are poorly immunogenic and remain largely unex-

Table 2 Shared antigens presented by HLA-A2 in two unrelated lines, SCLC#2 and SCLC#7

CTLs were generated from purified CD8 cells, as described in the text, using the stimulator cells indicated. Individual CTL lines were tested against either A2-transfected SCLC line in Cr-release assays for 4 h. Lysis was scored positive when the unrelated target showed 80% or more of the lysis compared with lysis of the stimulator cell.

Stimulator cells	Target cells lysed/CTL lines tested	
	SCLC#2-A2	SCLC#7-A2
SCLC#7-A2	3/4	4/4

Table 3 Infrequent antigen sharing between A1-transfected SCLC, non-small lung adenocarcinomas, and melanoma

CTLs were generated from purified CD8 cells, as described in the text, using the stimulator cells indicated. Individual CTL lines were tested against A1-transfected SCLCs, adenocarcinoma of the lung (AD), and melanoma (MEL) in Cr-release assays for 4 h. Lysis was scored positive when the unrelated target showed 50% or more of the lysis of the stimulator cell.

Stimulator cells	Target cells lysed/CTL lines tested			
	SCLC#2-A1	AD#100-A1	AD#101-A1	MEL113-A1
SCLC#2-A1	7/7	1/7	1/6	1/6

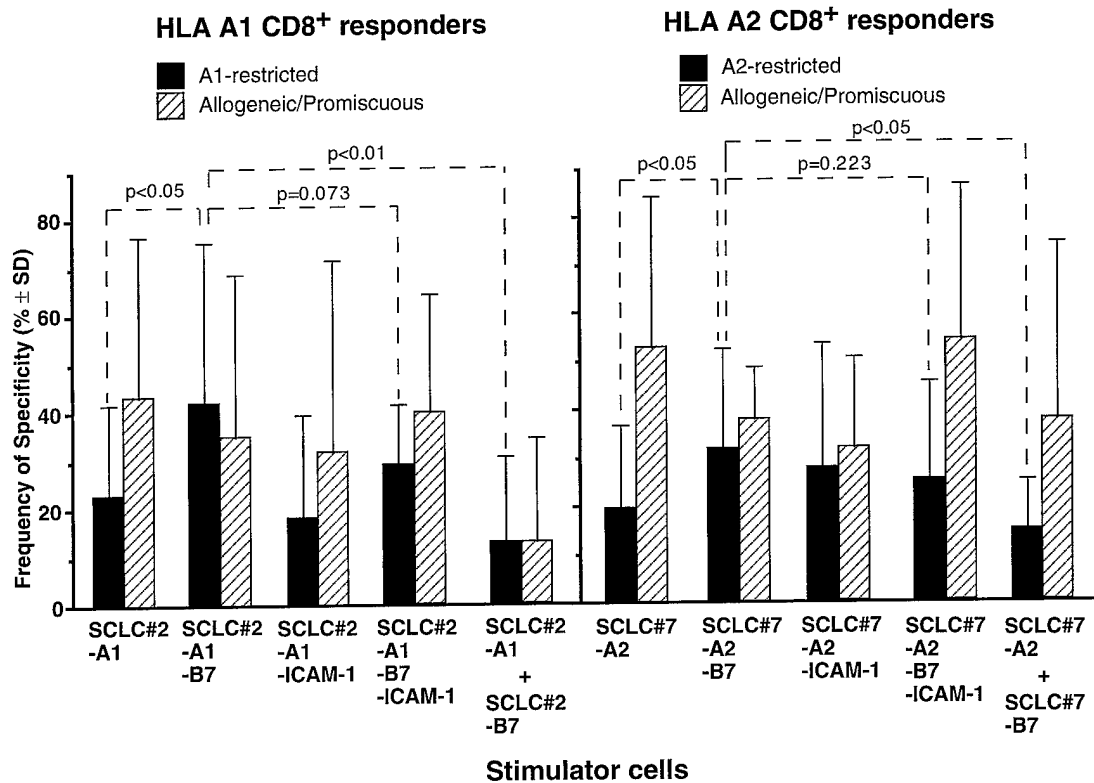


Fig. 5. Effect of B7.1 and ICAM-1 transfection on the frequency in generation of HLA-restricted CTLs. A1 and A2 responder CD8 cells were stimulated with class I-matched tumor transfectants and with double and triple transfectants, as indicated on the *abscissa* of the graph. In addition, stimulation was carried out by mixing class I-transfected and B7.1-transfected cells with CD8 responder cells. To evaluate MHC restriction, all wells were analyzed against four targets at an E:T ratio of 10:1; the four targets were SCLC#2 (mock), SCLC#2-HLA-A1, SCLC#2-HLA-A2, and K562 (left panel) or SCLC#7 (mock), SCLC#7-HLA-A1, SCLC#7-HLA-A2, and K562 (right panel). Results are means of 12 experiments for HLA-A1 CTL response and eight experiments for HLA-A2 CTL response; bars, SD. Significance values were calculated according to the Student *t* test. Class I-restricted CTLs lyse the appropriate target at least 1.3 times more than mismatched or wild-type targets.

explored because of the difficulty of generating CTL responses against these tumors. It is generally accepted that CD8⁺ T cells physiologically are activated by APCs with CD4 help in a pathway known as cross-priming or indirect antigen presentation. Indirect antigen presentation, however, is not easily replicated *in vitro* and does not lend itself to the discovery of MHC-restricted tumor antigens. It is, therefore, desirable to devise a method of direct antigen presentation that

allows the exploration of the important field of tumor antigenicity in poorly immunogenic solid tumors.

In this report, the response of purified human CD8⁺ T cells to transfected SCLC cells matched at one MHC class I locus is analyzed. We demonstrate the presence of shared, HLA-A1- or HLA-A2-restricted antigens in addition to private, not shared antigens in two unrelated SCLC lines. HLA-A1 or HLA-A2 transfection and expres-

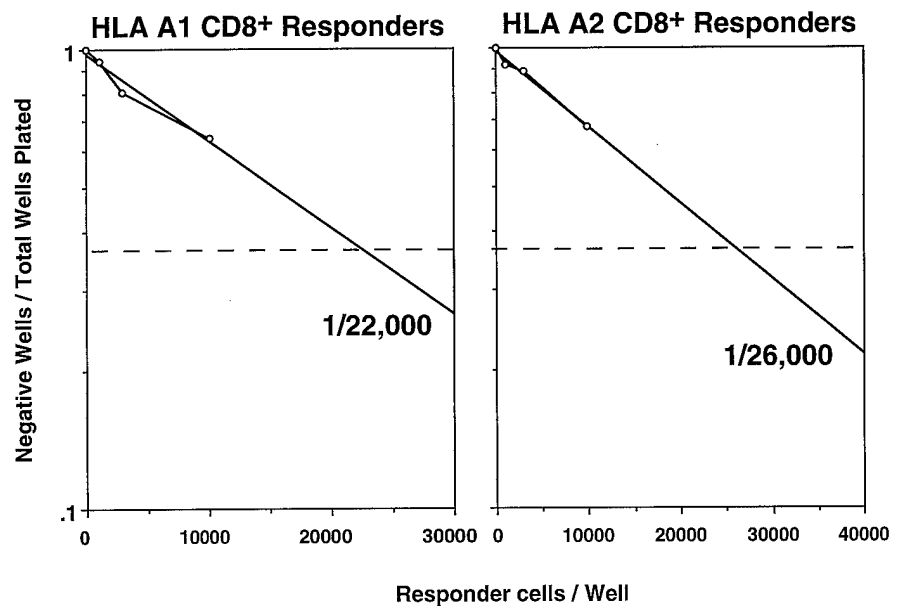


Fig. 6. CTLp frequencies of CD8⁺ T cells of two healthy volunteers for SCLC#2-HLA-A1 and SCLC#7-HLA-A2.

sion of tumors are critical for the generation of HLA-A1- or HLA-A2-restricted CTL responses. Single-cell cloning confirmed that microcultures contained the relevant MHC class I-restricted CTLs, along with allogeneic and promiscuous CTLs. Coulie *et al.* (56) and Mazzocchi *et al.* (54) showed that CTLp frequencies for autologous melanoma cells ranged from 1/720 to 1/33,000 in PBLs of melanoma patients. It is likely that, in some melanoma patients, expansion of CTLp takes place and may explain the high frequency in some patient samples. CTLp frequencies of 1/24,000 in our study are derived from healthy volunteers and represent the unexpanded, HLA-A1- or HLA-A2-restricted CD8⁺ CTLp repertoire for the two SCLC lines.

What is the nature of the tumor antigen recognized by the CTLs? About 75% of the MHC-restricted, SCLC-specific CTL lines recognize antigens shared by the two SCLC lines used, whereas the other 25% recognize private antigens. The molecular nature of the antigens is not known presently, and efforts are under way using these CTLs to define the antigen(s). By analogy to melanomas (17, 20, 21, 27, 28, 57), the most likely interpretation of our data would suggest that SCLCs express both shared and private tumor antigens. The shared antigens may represent unmutated embryonal antigens, whereas the private antigens may come from specific mutations.

For the generation of CTLs in the direct antigen stimulation mode, costimulation is very important. Suboptimal costimulation or culture conditions resulted in lower apparent CTLp frequencies and increased frequencies of allogeneic responses. Overgrowth of allogeneic CTLs is not a problem in the culture systems described. This is attributable largely to MHC class I transfection and high level expression by the tumor. Equally important is the omission of APCs and CD4 T cells in primary stimulation, reducing allogeneic stimulation. To obtain MHC-restricted CTLs, it was important to use highly purified CD8 cells and to exclude CD8^{dim} NK cells. IFN- γ (58) or IL-12 had no further effect on CTL generation in our system (data not shown), suggesting that class I expression is not limiting after transfection of the tumor with HLA. Limiting dilution cloning of CTLs confirmed the specificity that had been deduced.

B7.1 expression on the tumor cells, in conjunction with MHC class I, effectively bypassed the need for CD4⁺ helper T cells in the generation of CD8⁺ CTLs. The molecular mechanism of CD4⁺ helper T cells for the generation of CD8⁺ CTLs has recently been clarified. CD40-Ligand expressed by CD4⁺ helper T cells interacts with CD40 on APCs and up-regulates B7, which is used as costimulus for CD28 signaling of CD8⁺ T cells (37–40). In agreement with the proposed function of CD40-Ligand, our analysis suggests that overexpression of B7.1 and MHC class I on tumor cells is sufficient to activate CD8⁺ CTLs directly and to bypass the need for APCs.

Although this study focused on MHC class I-restricted CD8⁺ CTL responses to SCLCs, we have generated similar data also for HLA-A1 and B7.1-transfected non-SCLCs,⁵ and we suggest that this concept may be generalizable to other tumors. Our studies indicate that matching of MHC class I alleles between the transfected tumor cells and the responder CD8⁺ T cells is an important factor for generation of CTLs with defined MHC class I restriction. Although HLA-A1 and HLA-A2 are suitable for this purpose, other alleles could also be used and investigated in a similar strategy. Methods to identify tumor antigens rely on the generation of MHC class I-restricted CTLs. Using partial MHC class I matching and direct stimulation of purified CD8⁺ T cells opens a novel avenue in this effort.

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⁵ Unpublished observations.

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